Induction of calcium currents by the expression of the α_1-subunit of the dihydropyridine receptor from skeletal muscle

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THE dihydropyridine (DHP) receptor purified from skeletal muscle comprises five protein subunits (α_1, α_2, β, γ and δ) and produces Ca^{2+} currents that are blocked by DHPs[1]. Cloning of the α_1- and α_2-subunits[2,3], the former affinity-labelled by DHP, has shown that the α_1-subunit is expressed in skeletal muscle alone, whereas the α_2- and δ-subunits are also expressed in other tissues[2,4,5]. Although the transient expression of the α_1-subunit in myoblasts from dysgenic mice (but not in oocytes) has been demonstrated[6], the use of these expression systems to determine the function of the α_1-subunit is complicated by the presence of endogenous Ca^{2+} currents[7,8], which may reflect the constitutive expression of proteins similar to the α_2-, β-, γ- and/or δ-subunits. We therefore selected a cell line which has no Ca^{2+} currents or α_2-subunit, and probably no δ-subunit for stable transformation with complementary DNA of the α_1-subunit. The transformed cells express DHP-sensitive, voltage-gated Ca^{2+} channels, indicating that the minimum structure of these channels is at most an $\alpha_1\beta\gamma$ complex and possibly an α_1-subunit alone.

We co-transfected murine L cells with the thymidine kinase selection gene (*tk*) and pDHPR α_1.tot, a plasmid containing the complete open reading frame of the cDNA of the skeletal muscle dihydropyridine receptor (DHPR) α_1-subunit[2,3] under the control of a metallothionein promoter[9]. RNAs from 7 out of 20 cloned cell lines were positive for DHPR α_1 messenger RNA and the three lines giving the most intense signals (Fig. 1*a*), were expanded further. These showed low but significant specific DHP binding (Fig. 1*b*), characterized by a K_d of 0.4 nM in LCa.11 cells and a maximum specific-binding capacity of 50–60 fmol mg^{-1} membrane protein (data not shown). This contrasts with 500–1,000 fmol per mg of cardiac sarcolemmal membranes and 15,000–80,000 fmol per mg of T-tubule membrane[10,1]. On average, each LCa.11 cell expresses 600–1,000 DHP-binding sites. Significantly, *tk*$^-$ L cells or LEM5 cells, transfected with the same expression plasmid[9] but containing the open reading frame of the M5 muscarinic acetylcholine receptor[11], showed no DHP binding.

An immunoblot analysis for the presence of α_1 in LCa.11 cells[12], revealed a band corresponding to a relative molecular mass (M_r) of 195,000 (195K) (Fig. 2). This is greater than the apparent M_r of the T-tubule α_1-subunit (170K), indicating that either the L cell processes the α_1-subunit differently from the skeletal muscle cell, or that during isolation the skeletal muscle α_1-subunit is proteolytically cleaved. We noted, for instance, that LCa.11 cell α_1-subunit is easily cleaved to a major fragment migrating with M_r 170K, raising the possibility[13] that one of the smaller components is derived from the same precursor as the α_1 subunit.

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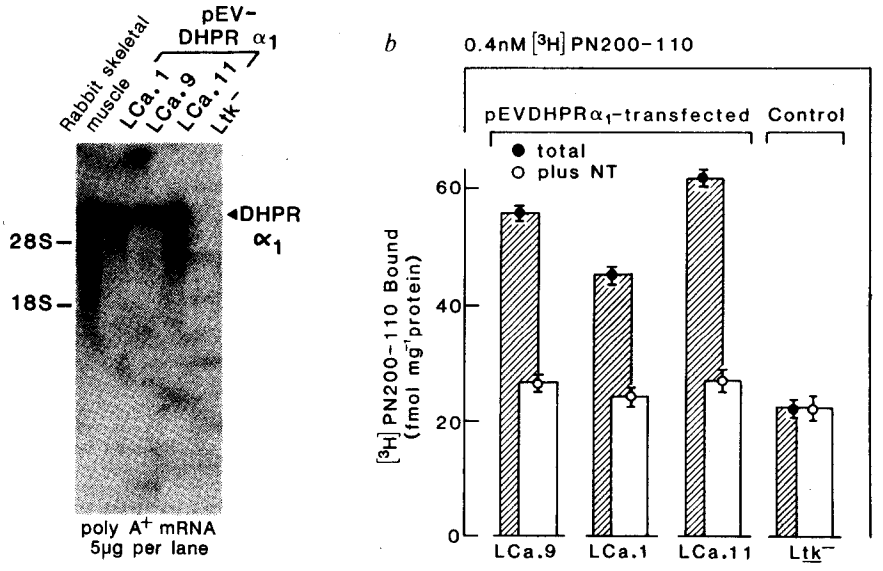

FIG. 1 Expression of DHPR α_1-mRNA and DHP-specific binding sites in L cells transfected with skeletal muscle DHPR α_1-cDNA. *a*, Hybridization blot analysis for the presence of DHPR α_1-mRNA in poly(A$^+$) mRNA isolated from rabbit skeletal muscle, from three cell lines stably transformed from *tk*$^-$ to *tk*$^+$ in the presence of pEV-DHPR α_1-DNA and from the recipient L*tk*$^-$ cell line. *b*, Binding of the dihydropyridine Ca^{2+}-channel antagonist [^{3}H]-(+)PN200-110 to membranes from stably transformed cells expressing DHPR α_1-mRNA, but not to membranes from control recipient L*tk*$^-$ cells. The skeletal muscle α_1-cDNA[2] was cloned from a rabbit skeletal-muscle cDNA libarary constructed in the Okayama–Berg pcD vector[20] by MacLennan *et al.*[21] and the complete open reading frame of the cDNA placed under the control of the mouse metallothionein-1 promoter in plasmid EV-142 (ref. 9) (gift from Dr R Palmiter, University of Washington), to give pEV-DHPR α_1.tot (E. P.-R. and L.B., unpublished observations). This plasmid was transfected by the calcium phosphate method[22] into murine thymidine kinase negative (*tk*$^-$) L cells[23]. Twenty individual cell clones were isolated with the aid of cloning rings after selection for *tk*$^+$ cells using media and conditions described previously[11] and expanded in minimum essential medium alpha medium (GIBCO) in the presence of 10% fetal bovine serum, penicillin/streptomycin and hypoxanthine, aminopterine and thymidine. Before collecting or testing, cells were plated at the equivalent density of $\sim 2 \times 10^6$ cells per 100-mm dish and grown to confluence (3 days) to densities of $\sim 10 \times 10^6$ cells per 100-mm dish. After a medium change and addition of 100 μM $ZnSO_4$ for 15 h to induce maximal DHPR α_1-mRNA levels and its translation, the cells were either collected for analysis of mRNA, DHP binding to membranes or immunoblotting of membrane proteins, or used for electrophysiological analysis of Ca^{2+} channel activities.

METHODS. RNA analysis: Poly(A$^+$) RNA was isolated from total RNA prepared by the guanidine-isothiocyanate/CsCl method using oligo (dT)-cellulose chromatography and electrophoresed through a 1.2% agarose gel in the presence of 50 mM sodium phosphate (P_i) buffer, pH 7.0, and 6.7% formaldehyde. The RNAs were transferred to Hybond-N membranes (Amersham). The membrane was then dried for 2 h at 80 °C, soaked in 6 × SSC, 5 × Denhardt's (1 × Denhardt's: 0.02% Ficoll, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone), 0.3 M P_i, pH 7.0, 0.5% SDS, 100 μg ml^{-1} sheared herring sperm DNA and 20% formamide for 4 h at 37 °C, and hybridized overnight at the same temperature in the same medium with 10^7 c.p.m. ml^{-1} of nick-translated *Sac*I(650)/*Sac*I(5313) (ref.3) fragment of the DHPR α_1-cDNA (specific activity 2.5×10^8 c.p.m. μg^{-1}). The membrane was then washed at increasing stringency ending with 0.1 × SSC plus 0.5% SDS at 62 °C, sealed into a polyethylene bag and autoradiographed for 30 h at −70 °C in the presence of two enhancing screens. For DHP-binding experiments, cells were induced with 100 μM $ZnSO_4$ for 15 h, rinsed twice with ice-cold 1 mM EDTA, 50 mM Tris-HCl, pH 7.2, scraped into ~4 ml of the rinsing medium with a rubber policeman, and homogenized in a Wheaton glass/teflon homogenizer (10 slow strokes at ~500 r.p.m.). The suspension was centrifuged at 420*g* for 5 min, the pellet was resuspended in 4 ml medium, rehomogenized and recentrifuged. The two low-speed supernatants were combined and centrifuged at 90,000*g* for 30 min. The pellet(membranes on figure) was resuspended in 50 mM Tris-HCl, pH 7.2, 1 mM EDTA. Typically, one 100 mm dish of confluent, Zn-treated cells yielded 500 μl of a membrane suspension with 1–1.5 mg protein ml^{-1} containing 60–70% of the specific binding sites. Binding assays were in a final volume of 1 ml at room temperature for 90 min in the presence of 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.2 and 0.4 nM [^{3}H]-(+)PN200-110 (8.0 Ci $mmol^{-1}$; Amersham). At the end of the incubation the samples were filtered under vacuum through Whatman GF/B glass fibre filters, washed 5 times with 5 ml of ice-cold 50 mM Tris-HCl, pH 7.2 with the aid of a Brandel filtration apparatus. Bound [^{3}H](+)PN200-110 was then determined by counting the radioactivity retained on the glass fibre filters using a liquid scintillation counter.

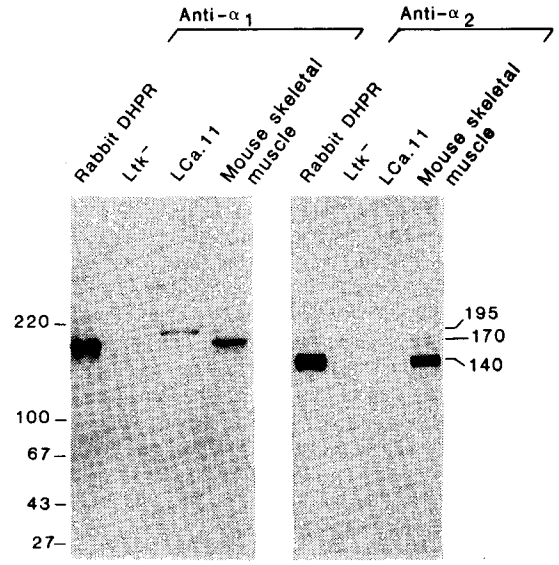

FIG. 2 Immunoblot analysis for presence of immunoreactive α_1- and α_2-proteins in membranes from LCa.11 cells and murine skeletal-muscle membranes, both positive for DHP binding, and in membranes from the recipient Ltk^- cells. Membranes from LCa.11 (600 μg per lane) and Ltk^- (600 μg per lane) cells were prepared as described above, except that the final resuspension was in a smaller volume to give concentrations of 15–30 mg ml^{-1}. Murine skeletal muscle membranes (50 μg per lane; 1.4 pmol DHP-binding sites per mg protein) were prepared as described in Knudson *et al.*[24], and pentameric rabbit skeletal muscle DHP-binding complex (2 μg per lane) was purified from triad membranes[13]. Membranes and purified protein were subjected to SDS–PAGE (3–12% gradient gels) using the Laemmli buffer system and then transferred to nitrocellulose according to Otter *et al.*[25]. Nitrocellulose blots were blocked with 150 mM NaCl, 50 mM P_i, pH 7.4, containing 5% (w/v) nonfat dry milk, and then incubated with either monoclonal antibody IIF7 directed against the DHPR α_1-protein (Anti-α_1) or affinity-purified polyclonal antibody GP5 directed against the α_2 protein (Anti-α_2). The specificities and properties of these antibodies have been described[5,12]. The transfers were then incubated with horseradish peroxidase-conjugated secondary antibodies, and developed using 4-chloro-1-napthol as substrate. Relative molecular mass standards ($\times 10^{-3}$) were prestained; calibrations are shown to the left and right.

By contrast, no endogenous protein reactive to α_2-subunit-specific guinea pig polyclonal antibody GP5 (ref. 5) was detected, although a positive reaction occurred in mouse skeletal muscle (Fig. 2). It seems therefore that the α_2-subunit, and probably the δ-subunit to which it is disulphide-bonded[1,4], are not present in the LCa.11 cells; the presence of the β- or γ-subunit has not been tested for specifically. Subunits α_2 and δ are glycosylated and retained on wheat germ agglutinin, together with their associated α_1- and β-components[1,14]. We tested whether L cells might have a functional homologue to the skeletal muscle α_2-subunit which would not cross react with the antibody, yet might interact with the α_1-subunit to form a complex which would be retained by a wheat germ agglutinin column. However, the LCa.11 α_1-subunit, when solubilized under conditions that lead to retention of the pentameric skeletal muscle or cardiac DHPR (A. T. Leung and K.P.C., unpublished observations), failed to be retained by wheat germ agglutinin (data not shown).

The three LCa cell lines, but not recipient tk^- L cells or control transfected LEM5 cells, expressed Ca^{2+} currents and LCa.11 cells produced the largest currents with the greatest consistency (Fig. 3). The only discernible currents in the Ltk^- and LEM5 cells have been inward and outward rectifying K^+ currents (data not shown). In the presence of Bay K 8644 (1–10 μM), 80% of LCa.11 cells with gigaohm input resistances gave rise to detectable Ca^{2+} tail currents. These currents were voltage-dependent, and were increased by the DHP agonist Bay K 8644 (ref. 15). The currents were blocked by Co^{2+} or Cd^{2+}, but tail currents of reduced amplitude persisted in the presence of Cd^{2+} (ref. 16). At voltages near threshold we occasionally observed single-channel events during the step and afterwards during the tail, and calculated from this a unitary conductance

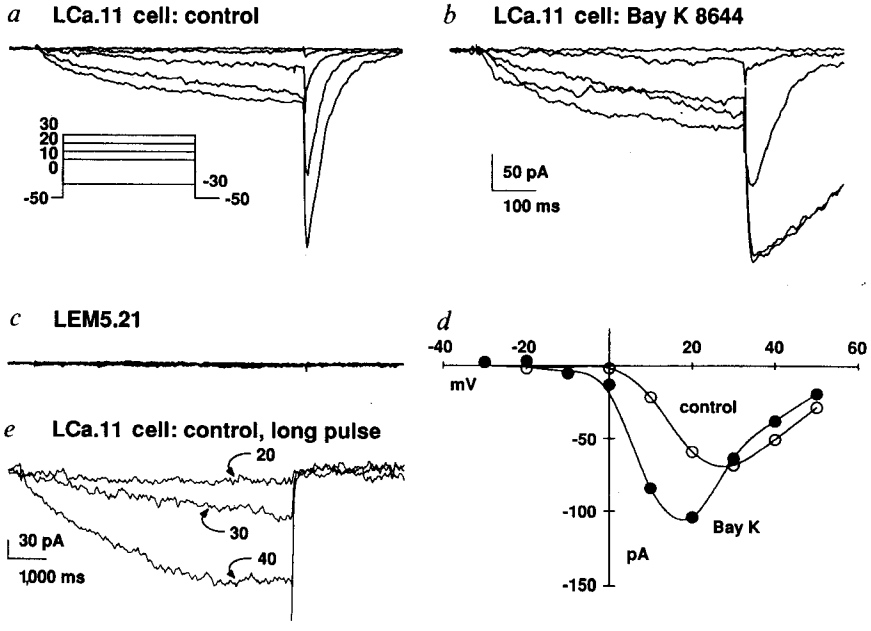

FIG. 3 Ca^{2+}-channel currents of an LCa.11 cell stably transformed with the DHPR α_1-subunit cDNA before (*a*) and after (*b*) addition of Bay K 8644, and current-voltage relationships derived therefrom (*d*). Bay K 8644 at 2 μM produces typical effects: an increase in peak currents and a hyperpolarizing shift in activation at lower potentials. Voltage control was lost during the two largest tail currents shown in *b*, possibly because of blockage of the pipette. As control, LEM5 cells were stably transformed in exactly the same manner, except that the coding sequence was for the rat M5 muscarinic acetylcholine receptor (*c*).
METHODS. The gigaseal patch clamp method was used to measure currents[26]. The second and third peak current-voltage relationships were obtained before (*a*) and after (*b*) the addition of (−) Bay K 8644 at 2 μM. The first and second I–V curves during control were the same. The cell was held at −50 mV and the 300-ms pulses shown in the insert of *a* were delivered at 0.1 Hz. The same protocol was used in *c*. In *e*, pulses were 7 s in duration and the currents displayed were produced by test pulses to 20, 30 and 40 mV delivered at intervals of one minute. Bath solution contained (in mM): 115 $BaCl_2$, 10 HEPES (pH adjusted to 7.4 with N-methyl-D-glucamine (NMG)) and Ba^{2+} was the charge carrier through the Ca^{2+} channels in all experiments. Pipette solution was (in mM): 130 NMG, 15 EGTA, 5 BAPTA, 11.5 $MgCl_2$, 8 $CaCl_2$, 3 Na_2ATP, 0.1 Na_2GTP (pH adjusted to 7.2 with MES). Current records were corrected for linear capacitive and leak currents online by a P/4 procedure with four 0.25 amplitude-inverted steps from the holding potential of −50 mV and preceding the depolarizing step. Records in *a*, *b*, and *c* were filtered to −3dB at 300 Hz and digitized at 600 Hz to 1.7 kHz; in *e* the corner frequency f_c was 6 Hz and the sampling rate was 50 Hz. For the experiments using inorganic blockers, pulses were 630 ms in duration. Co^{2+} at 10 mM and Cd^{2+} at 5 mM were added as chloride salts to a static bath and blocked all components of Ba^{2+} current during the voltage step, revealing outward currents probably carried by Cs^+ through K^+ channels. Larger concentrations of these blockers were necessary in the presence of isotonic Ba^{2+}.

of 6 pS, which compares reasonably well with the majority value of 10 pS recorded under similar ionic conditions for Ca^{2+} channels of skeletal-muscle T-tubules incorporated into planar lipid bilayers[17]. From these tail-current amplitudes recorded with satisfactory voltage control, we estimated at least 400–800 channels per cell, which agrees with the estimated number of binding sites.

The Ca^{2+} currents rose very slowly (Fig. 3*e*). The fastest half-times (~500 ms) were considerably slower than the activation half-times (~50 ms) of Ca^{2+} currents in skeletal muscle[18] and phenotypic cell lines of skeletal muscle[19], which are themselves much slower than the onset of Ca^{2+} currents in neurons, cardiac and smooth muscle cells. Deactivation of the tail currents reflects the slowness of the activation process and inactivation (not shown) is slower still.

Our results show that the protein encoded by the α_1-subunit cDNA is the DHP receptor and confirm the notion that this subunit is directly involved in Ca^{2+} permeation[6]. Moreover, it seems that the α_1-subunit can function as a Ca^{2+} channel in the absence of the α_2- and probably δ-subunits; indeed the α_1-subunit alone may suffice for these functions, although this has yet to be determined.

Perhaps as important are our findings that the transfected protein was significantly larger than rabbit skeletal-muscle protein and produced much slower Ca^{2+} currents. This could reflect an intrinsic deficiency in the processing capacity of the L cell. Another possibility, however, based on the finding that the α_1-subunit is purified from its natural T-tubule environment along with the α_2-, β-, γ- and δ-subunits, is that to acquire 'skeletal muscle' properties, α_1 must associate with one or more of these components which may be missing in LCa . 11 cells.

The present report demonstrates that stable expression of voltage-gated Ca^{2+} channels in tissue culture cells is possible. This adds stable transformation of cells, excitable or non-excitable, to the growing list of methods for studying the structure and function of voltage-gated ion channels. □

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